PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of
Daniel B. Drachman

Group Art Unit: 1633

Serial No.: 09/205,096

Examiner: E. Sorbello

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Attorney Docket No.: 01107.77737

For: TARGETING ANTIGEN-SPECIFIC CELLS FOR SPECIFIC IMMUNOTHERAPY OF AUTOIMMUNE DISEASE

DECLARATION UNDER 37 C.F.R. RULE 1.132

Honorable Assistant Commissioner
for Patents
Washington, DC 20231

Sir:

I, Daniel B. Drachman, hereby declare:

1. THAT, I am the inventor of the invention described and claimed in the application named above. I am a medical doctor. Presently, I am a full professor of Neurology and Neurosciences and the Director of the Neuromuscular Clinic and Research Laboratory at Johns Hopkins University School of Medicine.

2. THAT, we have demonstrated experimentally that antigen presenting cells ("APCs") transduced by vaccinia virus vectors ("VVV") engineered to express acetylcholine receptors ("AChR"), or AChR and Fas ligand ("FasL"), or AChR and FasL and truncated FADD, caused effective and specific killing of AChR-specific T-cells in culture.

3. THAT, the experimental system employed standard and well-known methods and materials which are also described in the captioned patent application.

Briefly, a first genetic element contained the extracellular domain of the α subunit of Torpedo AChR (amino acids 1-210) functionally connected to the mouse LAMP1 signal sequence and a transmembrane/cytoplasmic tail. A second genetic element

encoding FasL was optionally inserted into VVV. A third element encoding truncated FADD was optionally inserted into VVV. The elements, alone or in various combinations, were inserted into VVV by homologous recombination. The VVV employed was the wt WR strain from ATCC which was attenuated by psoralen UV irradiation. Radio-labeled T-cell lines specific to AChR and/or keyhole limpet hemocyanin ("KLH") were produced by methods known in the art (stimulation by AChR or KLH, respectively) from Lewis rats and from lpr/lpr mutant rats (which do not express Fas and thus are not succeptable to FasL). Splenocytes from naïve mice or rats were used as APCs and were transduced with engineered or wt VVVs. The APC were tested to determine their effect on T-cells by co-incubation with the T-cells. Various controls, such as added purified acetylcholine receptor, were employed. As shown in Figure 3B (Exhibit A), mouse splenocytes transduced with the three genetic elements had a powerful destructive effect on the target cells (lymphoma cells). The effect was not seen when the genetic elements were used singly. Moreover, the effect was not seen when the target cells did not express Fas (lpr/lpr mice) as shown in Figure 6 (Exhibit A).

4. THAT, the data demonstrate that T-cells were targeted by the AChR extracellular domain and the killing was specifically mediated by FasL. The killing was very effective, because, after exposure to the relevant engineered APCs, no stimulation by added purified AChR receptor of T-cells specific for AChR was observed. The results indicate that the extracellular domain of the α subunit of AChR was sufficient to target virtually the entire population of AChR stimulated T-cells.

5. THAT, in a separate set of experiments, we have demonstrated that APCs transduced with VVV engineered to express influenza hemagglutinin ("HA") and FasL effectively and specifically killed HA-specific T-cells in culture. The materials and methods were similar to those described in the captioned patent application and paralleled the experimental approach described in paragraph 3, *supra*. VVV were engineered to express combinations of the HA gene, FasL gene, and/or truncated FADD gene. HA-specific T cells were used. Ovalbumin ("OVA")-specific T-cells were used as control cells. As shown in Figure 4 (Exhibit B), HA-specific T-cells were specifically and strongly growth inhibited by the triple gene construct containing HA, FasL, and truncated

FADD. T-cells of different specificity (OVA) were not inhibited by the same construct. When apoptosis was measured rather than mere growth inhibition, it was clear that the triple gene construct induced apoptosis far more effectively than anti-Fas antibody. See Figure 5 (Exhibit B). Thus, the triple gene therapy inhibits growth of specific T-cells and induces apoptosis of the specific T-cells.

6. THAT the data demonstrate that the VVV-transferred HA gene stimulated and targeted HA-specific T- cells, that FasL was effectively expressed from the VVV vectors, that effective HA-specific T-cells killing was specifically mediated by FasL. The killing was very effective and only HA-specific T-cells were targeted.

7. THAT the observations in paragraphs 3-6 demonstrate that the methods of the captioned application lead to effective presentation of antigens by APCs, and effective targeting and destruction of specific T-cells. Two different antigens were successfully tested according to the method of the invention.

8. THAT, all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 4/24/01

Daniel B. Drachman

EXHIBIT A

Legends:

Figure 3B Functional effect of FasL:

Balb/C mouse splenocytes were transduced by overnight infection with various attenuated VVV preparations @ 20 pfu/cell. A20 target cells were pre-labeled with [^{3}H]-TdR. The transduced splenocytes were incubated overnight with A20 target cells at different Effector:Target (E:T) ratios. Triplicate wells, each containing 2.5 x 10^4 A20 cells/well were used. APCs transduced with the "triple gene" VVV induced marked loss of radioactivity. APCs transduced with control VVV expressing TrFADD or Sig-AChR-LAMP1, or infected with wt VV - but without FasL - did not.

Abbreviations:

wt - APCs with wild type vaccinia virus.
TrFADD - APCs transduced with VVV expressing truncated FADD.
TAChR - APCs transduced with VVV expressing the Sig-AChR-LAMP1 gene construct.

Figure 6. Essential role of FasL in elimination of AChR-specific T cells by "3-gene" transduced APCs.

AChR-specific T cell lines were prepared from lymph node cells of C57B1/6 mice or lpr/lpr mutant mice (lacking Fas), that were immunized with AChR. Spleen cells from naive C57B1/6 mice were used as APCs. They were transduced with Sig-AChR-LAMP1 VVV (TAChR); or the AChR "3-gene" VVV (TAChR-FasL-TrFADD); or infected with attenuated control vaccinia (wt). AChR-specific T cells (5 x 10^4) from C57B1/6 mice, or from lpr/lpr mice, were incubated with 2.5 x 10^5 APCs in triplicate microwells for 5 days, and [^{3}H]-TdR was added for the final 8 h of the culture. The AChR-specific T cells from lpr mice proliferated in response to the "3 gene" VVV transduced APCs. The AChR-specific T cells from C57B1/6 mice were inhibited.

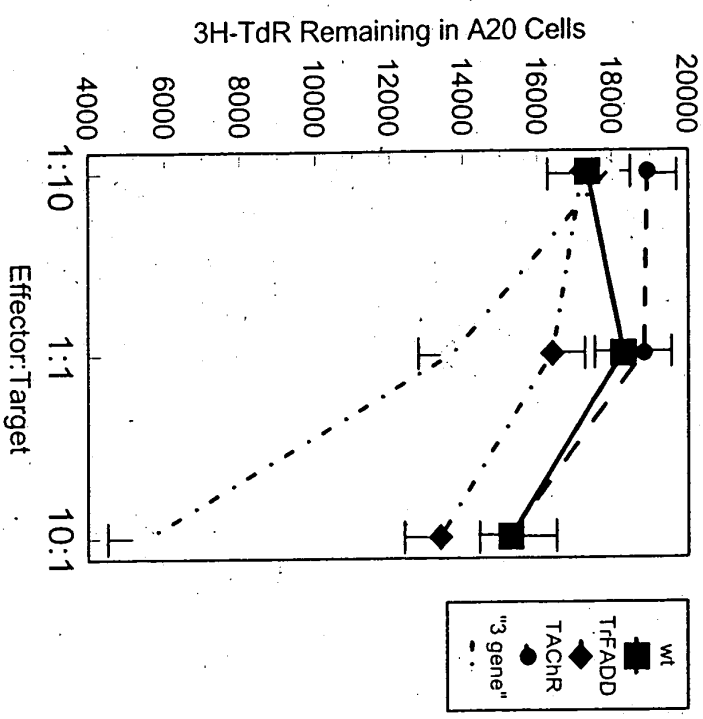
3H-TdR Remaining in A20 Cells
4000
6000
8000
10000
12000
14000
16000
18000
20000
1:10
1:1
10:1
Effector:Target
wt
TrFADD
TAChR
"3 gene"

FIGURE 3B

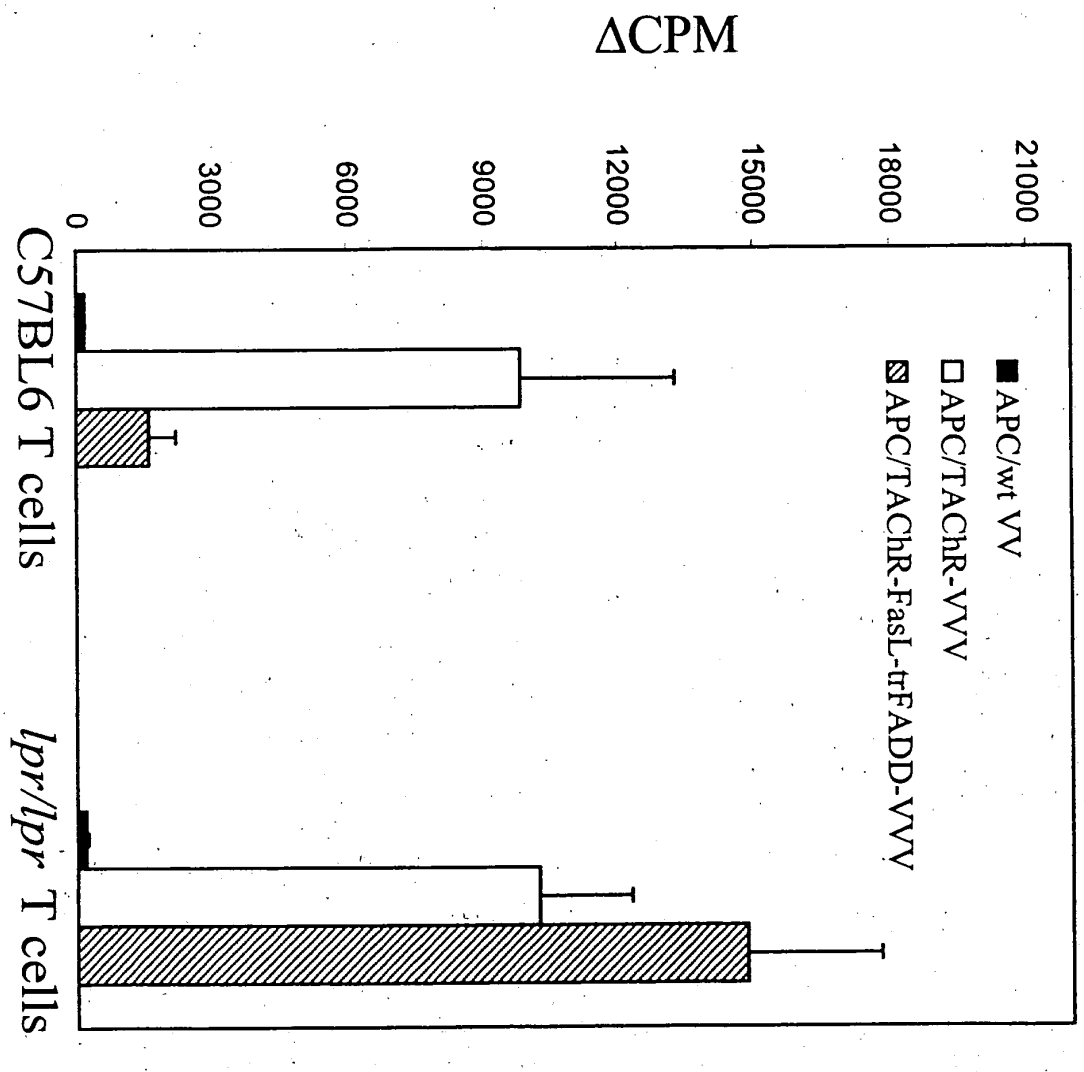
ΔCPM
21000
18000
15000
12000
9000
6000
3000
0
APC/wt VV
APC/TAChR-VVV
APC/TAChR-FasL-trFADD-VVV
C57BL6 T cells
lpr/lpr T cells

FIGURE 6

EXHIBIT B

Figure Legends:

Figure 4 Antigen specificity of effect by vvv-transduced APCs.

Lymph node cells from HA-specific TCR transgenic mice, and from OVA-specific TCR transgenic mice were pre-stimulated in separate bulk cultures with HA peptide (10 μg/ml) or OVA (40 μg/ml) respectively for 48 hours. HA-specific or OVA-specific T cells were then co-cultured with Balb/C splenocytes that had been infected with either attenuated "3-gene" vvv or with control wt vv for 5 days (2×10^4 T cells; 2×10^5 APCs, in triplicate cultures), and pulsed for the last 18 h with [^{3}H]-TdR. Results are expressed as Δcpm ± SEM. Note marked inhibition of HA-specific T cells, with negligible effect on the OVA-specific T cells.

Figure 5. Antigen targeting of vvv transduced APCs enhances FasL effect.

Spleen cells from HA-specific TCR transgenic mice were used as target T cells. They were pre-stimulated for 48 h with HA peptide (10 μg/ml), and then co-cultured overnight with Balb/C APCs infected with various attenuated vvv, as indicated (5×10^4 target cells and 5×10^5 APCs, in triplicate microcentrifuge tubes). The supernatant was collected, and fragmentation of DNA indicative of apoptosis was determined by an ELISA method that detects histone-bound mono- and oligonucleotides (see text). The background was measured from supernatants of vvv-transduced APCs alone. The results, given as the "enrichment factor", are considered positive if they are significantly greater than 1. Note that co-culture with "triple-gene" transduced APCs induced a marked increase of DNA fragmentation; co-culture with APCs transduced with FasL and TrFADD did not.

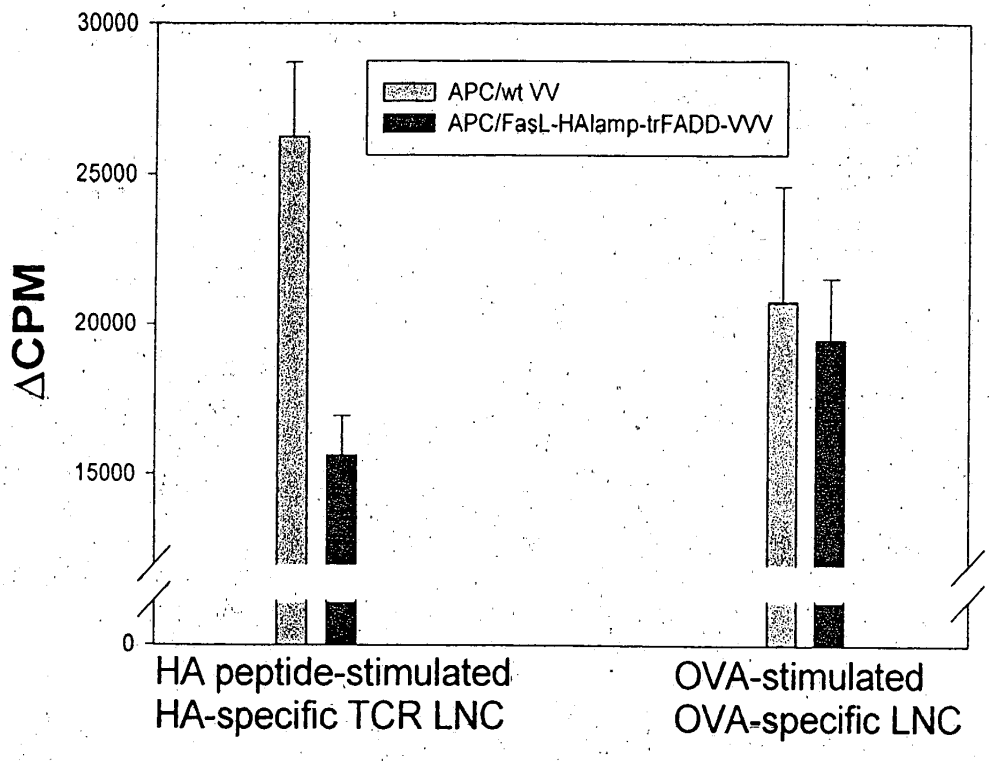
30000
25000
20000
15000
0
ΔCPM
APC/wt VV
APC/FasL-HAlamp-trFADD-VVV
HA peptide-stimulated
HA-specific TCR LNC
OVA-stimulated
OVA-specific LNC

FIGURE 4

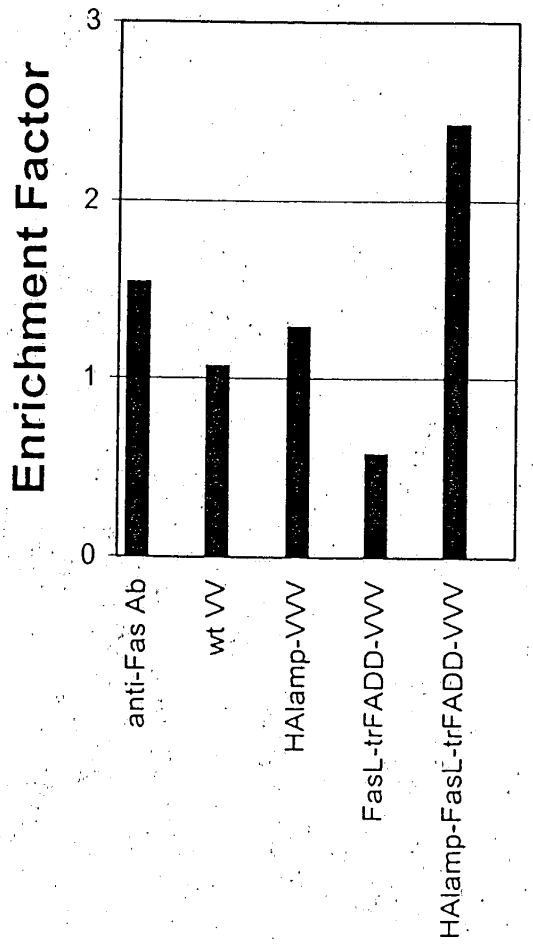
Enrichment Factor
3
2
1
0
anti-Fas Ab
wt VV
HAlamp-VVV
FasL-trFADD-VVV
HAlamp-FasL-trFADD-VVV

FIGURE 5